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(54) Title: TRANSFECTION AND TRANSFER OF MALE GERM CELLS FOR GENERATION OF TRANSGENIC SPECIES

(57) Abstract

A composition for in vivo transfection of vertebrate male germ cells comprises a nucleic acid or transgene, and a gene delivery system, and optionally a protective internalizing agent, such as an endosomal lytic agent, a virus or a viral component, which is internalized by cells along with the transgene and which enhances gene transfer through the cytoplasm to the nucleus of the male germ cell. A pharmaceutical preparation and a transfer kit utilize the composition. A method for introducing a polynucleotide into vertebrate male germ cells comprises the administration of the composition to a vertebrate. A method for isolating or selecting transfected cells utilizes a reporter gene, and a method for administering transfected male germ cells utilizes male germ cells which have been transfected in vitro.

TRANSFECTION AND TRANSFER OF MALE GERM CELLS FOR GENERATION OF TRANSGENIC SPECIES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of transgenics and gene therapy. More specifically, this invention relates to in vitro and in vivo methods for transfecting germ cells and, in some instances, incorporating a nucleic acid segment encoding a specific trait into the male germ cells of an animal. When the nucleic acid becomes incorporated into the germ cell genome, upon mating, or in vitro fertilization and the like, the trait may be transmitted to the progeny. The present technology is suitable for breeding progeny with or without a desired trait by modifying their genome. This technology is also suitable for use in introducing a therapeutic gene into the germ or support cells (e.g., Leydig or Sertoli cells) of the testis and is, therefore, suitable for use in gene therapy for males with fertility problems associated with genetic defects.

Description of the Background

The field of transgenics was initially developed to understand the action of a single gene in the context of the whole animal and phenomena of gene activation, expression, and interaction. This technology has been used to produce models for various diseases in humans and other animals. Transgenic technology is amongst the most powerful tools available for the study of genetics, and the understanding of genetic mechanisms and function. It is also used to study the relationship between genes and diseases. About 5,000 diseases are caused by a single genetic defect. More commonly, other diseases are the result of complex interactions between one or more genes and environmental agents, such as viruses or carcinogens. The understanding of such interactions is of prime importance for the development of therapies, such gene therapy and drug therapies, and also treatments such as organ transplantation. Such treatments compensate for functional deficiencies and/or may eliminate undesirable functions expressed in an organism. Transgenesis has also been used for the improvement of livestock, and for the large scale production of biologically active pharmaceuticals.

Historically, transgenic animals have been produced almost exclusively by micro injection of the fertilized egg. The pronuclei of fertilized eggs are micro injected in vitro with foreign, i.e. xenogeneic or allogeneic DNA or hybrid DNA molecules. The micro injected fertilized eggs are then transferred to the genital tract of a pseudopregnant female. The generation of transgenic animals by this technique is generally reproducible, and for this reason little has been done to improve on it. This technique, however, requires large numbers of fertilized eggs. This is partly because there is a high rate of egg loss due to lysis during micro injection. Moreover manipulated embryos are less likely to implant and survive in utero. These factors contribute to the technique's extremely low efficiency. For example, 300-500 fertilized eggs may need to be

extracting germ cells from the gonad of a suitable donor or from the animal's own gonad, using a novel isolation method, transfecting them in vitro, and then returning them to the testis under suitable conditions where they will spontaneously repopulate it. The ex vivo method has the advantage that the transfected germ cells may be screened by various means before being returned to the testis to ensure that the transgene is incorporated into the genome in a stable state. Moreover, after screening and cell sorting only enriched populations of germ cells may be returned. This approach provides a greater chance of transgenic progeny after mating.

This invention also relates to a novel method for the isolation of spermatogonia, comprising obtaining spermatogonia from a mixed population of testicular cells by extruding the cells from the seminiferous tubules and gentle enzymatic disaggregation. The spermatogonia or stem cells which are to be genetically modified, may be isolated from a mixed cell population by a novel method including the utilization of a promoter sequence, which is only active in cycling spermatogonia stem cell populations, for example, b-Myb or a spermotogonia specific promoter, such as the c-kit promoter region, c-raf-1 promoter, ATM (axataia-telangiectasia) promoter, RBM (ribosome binding motif) promoter, DAZ (deleted in azoospermia) promoter, XRCC-1 promoter, HSP 90 (heat shock gene) promoter, or FRMI (from fragile X site) promoter, optionally linked to a reporter construct, for example, the Green Fluorescent Protein Gene (EGFP). These unique promoter sequences drive the expression of the reporter construct only in the cycling spermatogonia. The spermatogonia, thus, are the only cells in the mixed population which will express the reporter construct and they, thus, may be isolated on this basis. In the case of the green fluorescent reporter construct, the cells may be sorted with the aid of, for example, a FACs scanner set at the appropriate wavelength or they may be selected by chemical methods.

This invention also relates to the repopulation of a testis with germ cells that have been isolated from a fresh or frozen testicular biopsy. These germ cells may or may not be genetically manipulated prior to reimplantation.

For transfection, the method of the invention comprises administering to the animal, or to germ cells in vitro, a composition comprising amounts of nucleic acid comprising polynucleotides encoding a desired trait. In addition, the composition comprises, for example, a relevant controlling promoter region made up of nucleotide sequences. This is combined with, for example, a gene delivery system comprising a cell transfection promotion agent such as retro viral vectors, adenoviral and adenoviral related vectors, or liposomal reagents or other agents used for gene therapy. These introduced under conditions effective to deliver the nucleic acid segments to the animal's germ cells optionally with the polynucleotide inserted into the genome of the germ cells. Following incorporation of the DNA, the treated animal is either allowed to breed naturally, or reproduced with the aid of assisted reproductive technologies, and the progeny selected for the desired trait.

This technology is applicable to the production of transgenic animals for use as animal models, and to the modification of the genome of an animal, including a human, by addition, modification, or subtraction of genetic material, often resulting in phenotypic changes. The present methods are also applicable to altering the carrier status of an animal, including a human, where that individual is carrying a gene for a recessive or dominant gene disorder, or where the individual is prone to pass a multigenic disorder to his offspring.

Animals that were shown to carry suitably modified sperm cells then may be either allowed to mate naturally, or alternatively their spermatozoa are used for insemination or in vitro fertilization. The thus obtained transgenic progeny may be bred, whether by natural mating or artificial insemination, to obtain further transgenic progeny. The method of this invention has a lesser number of invasive procedures than other available methods, and a high rate of success in producing incorporation into the progeny's genome of the nucleic acid sequence encoding a desired trait.

Primordial germ cells are thought to arise from the embryonic ectoderm, and are first seen in the epithelium of the endodermal yolk sac at the E8 stage. From there they migrate through the hindgut endoderm to the genital ridges. The primitive spermatogonial stem cells, known as A0/As, differentiate into type B spermatogonia. The latter further differentiate to form primary spermatocytes, and enter a prolonged meiotic prophase during which homologous chromosomes pair and recombine. Several morphological stages of meiosis are distinguishable : preleptotene, leptotene, zygotene, pachytene, secondary spermatocytes, and the haploid spermatids. The latter undergo further morphological changes during spermatogenesis, including the reshaping of their nucleus, the formation of acrosome, and assembly of the tail. The final changes in the spermatozoon take place in the genital tract of the female, prior to fertilization. The uptake of the nucleic acid segment administered by the present in vivo method to the gonads will reach germ cells that are at one or more of these stages, and be taken up by those that are at a more receptive stage. In the ex vivo (in vitro) method of genetic modification, generally only diploid spermatogonia are used for nucleic acid modification. The cells may be modified in vivo using gene therapy techniques, or in vitro using a number of different transfection strategies.

The inventors are, thus, providing in this patent a novel and unobvious method for; isolation of male germ cells, and for the in vivo and ex vivo (in vitro) transfection of allogeneic as well as xenogeneic DNA into an animal's germ cells. This comprises the administration to an animal of a composition comprising a gene delivery system and at least one nucleic acid segment, in amounts and under conditions effective to modify the animal's germ cells, and allowing the nucleic acid segment to enter, and be released into, the germ cells, and to integrate into their genome.

The in vivo introduction of the gene delivery mixture to the germ cells may be accomplished by direct delivery into the animal's testis (es), where it is distributed to male germ cells at various stages of development. The in vivo method utilizes novel technology, such as injecting the gene delivery mixture either into the vasa efferentia, directly into the seminiferous tubules, or into the rete testis using, for example, a micropipette. To ensure a steady infusion of the gene delivery mixture, under pressures which will not damage the delicate tubule system in the testis, the injection may be made through the micropipette with the aid of a picopump delivering a precise measured volume under controlled amounts of pressure. The micropipette may be made of a suitable material, such as metal or glass, and is usually made from glass tubing which has been drawn to a fine bore at its working tip, e.g. using a pipette puller. The tip may be angulated in a convenient manner to facilitate its entry into the testicular tubule system. The micropipette may be also provided with a beveled working end to allow a better and less damaging penetration of the fine tubules at the injection site. This bevel may be produced by means of a specially manufactured grinding apparatus. The diameter of the tip of the pipette for the in vivo method of injection may be about 15 to 45 microns, although other sizes may be utilized

agent and the DNA utilized. This proportion is not crucial.

"Transfecting agent", as utilized herein, means a composition of matter added to the genetic material for enhancing the uptake of exogenous DNA segment(s) into a eukaryotic cell, preferably a mammalian cell, and more preferably a mammalian germ cell. The enhancement is measured relative to the uptake in the absence of the transfecting agent. Examples of transfecting agents include adenovirus-transferrin-polylysine-DNA complexes. These complexes generally augment the uptake of DNA into the cell and reduce its breakdown during its passage through the cytoplasm to the nucleus of the cell. These complexes may be targeted to the male germ cells using specific ligands which are recognized by receptors on the cell surface of the germ cell, such as the c-kit ligand or modifications thereof.

"Virus", as used herein, means any virus, or transfecting fragments thereof, which may facilitate the delivery of the genetic material into male germ cells. Examples of viruses which are suitable for use herein are adenoviruses, adeno-associated viruses, retroviruses such as human immune-deficiency virus, lentiviruses, such as Moloney murine leukemia virus and the retrovirus vector derived from Moloney virus called vesicular-stomatitis-virus-glycoprotein (VSV-G)-Moloney murine leukemia virus, mumps virus, and transfecting fragments thereof, and other viral DNA segments that facilitate the uptake of the desired DNA segment by, and release into, the cytoplasm of germ cells and mixtures thereof. The mumps virus is particularly suited because of its affinity for immature sperm cells including spermatogonia. All of the above viruses may require modification to render them non-pathogenic or less antigenic. Other known vector systems, however, may also be utilized within the confines of the invention.

"Genetic material", as used herein, means DNA sequences capable of imparting novel genetic modification(s), or biologically functional characteristic(s) to the recipient animal. The novel genetic modification(s) or characteristic(s) may be encoded by one or more genes or gene segments, or may be caused by removal or mutation of one or more genes, and may additionally contain regulatory sequences. The transfected genetic material is preferably functional, that is it expresses a desired trait by means of a product or by suppressing the production of another. Examples of other mechanisms by which a gene's function may be expressed are genomic imprinting, i.e. inactivation of one of a pair of genes (alleles) during very early embryonic development, or inactivation of genetic material by mutation or deletion of gene sequences, or by expression of a dominant negative gene product, among others.

In addition, novel genetic modification(s) may be artificially induced mutations or variations, or natural allelic mutations or variations of a gene(s). Mutations or variations may be induced artificially by a number of techniques, all of which are well known in the art, including chemical treatment, gamma irradiation treatment, ultraviolet radiation treatment, ultraviolet radiation, and the like. Chemicals useful for the induction of mutations or variations include carcinogens such as ethidium bromide and others known in the art.

DNA segments of specific sequences may also be constructed to thereby incorporate any desired mutation or variation or to disrupt a gene or to alter genomic DNA. Those skilled in the art will readily appreciate that the genetic material is inheritable and is, therefore, present in almost every cell of future generations of the progeny, including the germ cells.

Among novel characteristics are the expression of a previously unexpressed trait, augmentation or

in vivo artificial means. Artificial means include, but are not limited to, artificial insemination, in vitro fertilization, cloning and embryo transfer, intracytoplasmic spermatozoal microinjection, cloning and embryo splitting, and the like. However, others may also be employed.

The transfection of mature male germ cells may be also attained utilizing the present technology upon isolation of the cells from a vertebrate, as is known in the art, and exemplified in Example 10. The thus isolated cells may then be transfected ex vivo (in vitro), or cryopreserved as is known in the art and exemplified in Example 11. The actual transsection of the isolated testicular cells may be accomplished, for example, by isolation of a vertebrate's testes, decapsulation and teasing apart and mincing of the seminiferous tubules. The separated cells may then be incubated in an enzyme mixture comprising enzymes known for gently breaking up the tissue matrix and releasing undamaged cells such as, for example, pancreatic trypsin, collagenase type I, pancreatic DNAse type I, as well as bovine serum albumin and a modified DMEM medium. The cells may be incubated in the enzyme mixture for a period of about 5 min to about 30 min, more preferably about 15 to about 20 min, at a temperature of about 33°C to about 37°C, more preferably about 36 to 37°C. After washing the cells free of the enzyme mixture, they may be placed in an incubation medium such as DMEM, and the like, and plated on a culture dish. Any of a number of commercially available transfection mixtures may be admixed with the polynucleotide encoding a desire trait or product for transfection of the cells. The transfection mixture may then be admixed with the cells and allowed to interact for a period of about 2 hrs to about 16 hrs, preferably about 3 to 4 hrs, at a temperature of about 33°C to about 37°C, preferably about 36°C to 37°C, and more preferably in a constant and/or controlled atmosphere. After this period, the cells are preferably placed at a lower temperature of about 33°C to about 34°C, preferably about 30-35°C for a period of about 4 hrs to about 20 hrs, preferably about 16 to 18 hrs. Other conditions which do not deviate radically from the ones described may also be utilized as an artisan would know.

The present method is applicable to the field of gene therapy, since it permits the introduction of genetic material encoding and regulating specific genetic traits. Thus, in the human, for example, by treating parents it is possible to correct many single gene disorders which otherwise might affect their children. It is similarly possible to alter the expression of fully inheritable disorders or those disorders having at least a partially inherited basis, which are caused by interaction of more than one gene, or those which are more prevalent because of the contribution of multiple genes. This technology may also be applied in a similar way to correct disorders in animals other than human primates. In some instances, it may be necessary to introduce one or more "gene(s)" into the germ cells of the animal to attain a desired therapeutic effect, as in the case where multiple genes are involved in the expression or suppression of a defined trait. In the human, examples of multigenic disorders include diabetes mellitus caused by deficient production of, or response to, insulin, inflammatory bowel disease, certain forms of atheromatus cardiovascular disease and hypertension, schizophrenia and some forms of chronic depressive disorders, among others. In some cases, one gene may encode an expressible product, whereas another gene encodes a regulatory function, as is known in the art. Other examples are those where homologous recombinant methods are applied to repair point mutations or deletions in the genome, inactivation of a gene causing pathogenesis or disease, or insertion of a gene that is expressed in a dominant negative manner, or alterations of regulating elements such as gene promoters,

EXAMPLES

TRANSFECTION OF MALE GERM CELLS IN VIVO

In Vivo Adenovirus-enhanced Transferrin-polylysine-mediated Delivery of Green Lantern Reporter Gene Delivery System to Testicular Cells

The adenovirus enhanced transferrin-polylysine-mediated gene delivery system has been described and patented by Curiel al. (Curiel D.T., et al. Adenovirus enhancement of transferrin-polylysine-mediated gene delivery, PNAS USA 88: 8850-8854 (1991). The delivery of DNA depends upon endocytosis mediated by the transferrin receptor (Wagner et al., Transferrin-polycation conjugates as carriers for DNA uptake into cells, PNAS (USA) 87: 3410-3414 (1990). In addition this method relies on the capacity of adenoviruses to disrupt cell vesicles, such as endosomes and release the contents entrapped therein. This system can enhance the gene delivery to mammalian cells by as much as 2,000 fold over other methods.

The gene delivery system employed for the in vivo experiments was prepared as shown in examples below.

Example 1: Preparation of Transferrin-poly-L-Lysine Complexes

Human transferrin was conjugated to poly (L-lysine) using EDC (1-ethyl-3-(3- dimethyl aminopropyl carbodiimide hydrochloride) (Pierce), according to the method of Gabarek and Gergely (Gabarek & Gergely, Zero-length cross-linking procedure with the use of active esters, Analyt. Biochem 185 : 131 (1990)). In this reaction, EDC reacts with a carboxyl group of human transferrin to form an amine-reactive intermediate. The activated protein was allowed to react with the poly (L-lysine) moiety for 2 hrs at room temperature, and the reaction was quenched by adding hydroxylamine to a final concentration of 10 mM. The conjugate was purified by gel filtration, and stored at -20° C.

Example 2: Preparation of DNA for In Vivo Trasfection

The Green Lantern-1 vector (Life Technologies, Gibco BRL, Gaithersberg, MD) is a reporter construct used for monitoring gene transfection in mammalian cells. It consists of the gene encoding the Green Fluorescent Protein (GFP) driven by the cytomegalovirus (CMV) immediate early promoter. Downstream of the gene is a SV40 polyadenylation signal. Cells transfected with Green Lantern-1 fluoresce with a bright green light when illuminated with blue light. The excitation peak is 490 nm.

Example 3: Preparation of Adenoviral Particles

Adenovirus dl312, a replication-incompetent strain deleted in the Ela region, was propagated in the Ela trans-complementing cell line 293 as described by Jones and Shenk (Jones and Shenk, PNAS USA (1979) 79: 3665-3669). A large scale preparation of the virus was made using the method of Mittereder and Trapnell

Table 1: Comparison of Lipofection & Adenovirus Enhanced Transferrin-polylysine Transfection of CHO Cells

Sample	Treatment	Luciferase Activity (RLU)
1	$1x10^7$ particles + 6ug CMV-Luc	486
2	2.5×10^7 particles + 6ug CMV-Luc	1,201
3	5.0×10^7 particles + 6ug CMV-luc	11,119
4	1×10^9 particles + 6ug CMV-Luc	2,003,503
5	Lipofection	1,108
6	Unmanipulated cells	155

Example 6: In Vivo Delivery of DNA to Animal's Germ Cells via Tranferrin-L-lysine-DNA-Viral Complexes

The GFP DNA-transferrin-polylysine viral complexes, prepared as described in Example 4 above, were delivered into the seminiferous tubules of three (3)-week-old B6D2F1 male mice. The DNA delivery by transferrin receptor-mediated endocytosis is described by Schmit et al. and Wagner et al. (Schmit et al., Cell 4: 41-51 (1986); Wagner, E., et al. PNAS (1990), (USA) 81: 3410-3414 (1990)). In addition, this delivery system relies on the capacity of adenoviruses to disrupt cell vesicles, such as endosomes and release the contents entrapped therein. The transfection efficiency of this system is almost 2,000 fold higher than lipofection.

The male mice were anesthetized with 2% Avertin (100% Avertin comprises 10 g 2,2,2-tribromoethanol (Aldrich) and 10 ml t-amyl alcohol (Sigma), and a small incision made in their skin and body wall, on the ventral side of the body at the level of the hind leg. The animal's testis was pulled out through the opening by grasping at the testis fat pad with forceps, and the vas efferens tubules exposed and supported by a glass syringe. The GFP DNA-transferrin-polylysine viral complexes were injected into a single vasa efferentia using a glass micropipette attached to a hand held glass syringe or a pressurized automatic pipettor (Eppendorf), and Trypan blue added to visualize the entry of the mixture into the seminiferous tubules. The testes were then placed back in the body cavity, the body wall was sutured, the skin closed with wound clips, and the animal allowed to recover on a warm pad.

Example 7: Detection of DNA and Transcribed Message

Nine (9) days after delivery of the genetic material to the animals' testis, two of the animals were sacrificed, their testes removed, cut in half, and frozen in liquid nitrogen. The DNA from one half of the tissues, and the RNA from the other half of the tissues were extracted and analyzed.

(a) Detection of DNA

The presence of GFP DNA in the extracts was tested 9 days after administration of the transfection mixture using the polymerase chain reaction, and GFP specific oligonucleotides. GFP DNA was present in the testes of the animals that had received the DNA complexes, but was absent from sham operated animals.

to the injection with 800 Rads of gamma irradiation. One mouse became sick and was sacrificed 48 hours after the injection. The testes from this mouse were dissected, fixed and processed for histology.

The two remaining males were bred with normal females as shown. After 4 months pups were born. Litters are currently being screened for the integration of the transgene.

Example 11: Preparation of a Cell Suspension from Testicular Tissue for Cryopreservation

A cell suspension was prepared from mice of different ages as described below.

Group I: 7-10 day olds

Group II: 15-17 day olds

Group III: 24-26 day olds

The mice's testes were dissected, placed in phosphate buffered saline (PBS) decapsulated, and the seminiferous tubules were teased apart. Seminiferous tubules from groups I and II were transferred to HEPES buffered culture medium (D-MEM) (Gibco-BRL, Life Technologies, Gaithesburg, MD 20884) containing 1mg/ml Bovine serum albumin (BSA) (Sigma, St. Louis, MO 63178) and Collagenase Type I (Sigma) for the removal of interstitial cells. After a 10 minute incubation at 33 °C, the tubules were lifted into fresh culture medium. This enzymatic digestion was not carried out on the testes from group I because of their fragility.

The tubules from group II and III mice or the whole tissue from group I mice were transferred to a Petri dish with culture medium and were cut into 0.1-1mm pieces using a sterile scalpel and needle. The minced tissue was centrifuged at 500 x g for 5 minutes and the pellet was resuspended in 1ml of enzyme mix. The enzyme mix was made up in D-DMEM with HEPES (GibcoBRL) and consisted of 1mg/ml bovine serum albumin (BSA) (Sigma, embryo tested), 1mg/ml collagenase I (Sigma) and 5 mg/ml bovine pancreatic trypsin (Sigma) and 0.1mg/ml deoxyribonuclease I (DN-EP, Sigma). The tubules were incubated in enzyme mix for 30 minutes at 33 °C. After the incubation, 1ml of medium was added to the mix and the cells were centrifuged at 500 x g for 5 min. The cells were washed twice in medium by centrifugation and resuspension. After the final wash the cell pellet was resuspended in 250μl of culture medium and counted.

Example 12: Cryopreservation of Methods for Testicular Cells

(a) Propanediol (PROH)-sucrose Method

Testicular cells from a total of 31 mice (age 8-12 weeks) were cryopreserved using 6 different freezing and thawing protocols. In addition to freezing cell supsensions, pieces of testicular tissue were frozen (see freezing method above). The cell suspension was prepared as described above.

The cell suspension was incubated in a buffer stock solution consisting of 80% phosphate buffered saline (PBS) and 20% human serum (SPR, Helsinki, Finland) for 5 minutes. The cells were then incubated in 1.5M PROH for 10 minutes, pelleted by centrifugation and resuspended in 1.5M PROH with 0.1M sucrose. The cell suspension was loaded into straws (0.25μm, Paillette, L'Aigle, France) or 1 ml cryogenic vials (Nunc cryotube). Samples were frozen in a controlled temperature freezing machine (Planer Kryo, Series III, Planer Biomed, Sunbury on Thames, UK). The samples were cooled at a rate of 2 °C/min to -8 °C, and seeded

fetal calf serum and 10% filtered glycerol was added to the cells to make up 90% of the total volume. The resuspension was incubated at 37°C for 10 min. The samples were placed in a -70°C freezer for 24 hours after which they were stored in liquid nitrogen.

The thawing procedure was the same as that described for the Glycerol yolk method.

(g) **Freezing Testicular Tissue**

The method used for freezing whole testicular tissue was the same as the method we described previously for freezing ovarian tissue (Hovatta, et al., Human Reprod. 11:1268-1272 (1996). The testicles of 6 mice were decapsulated in culture medium (D-MEM) and cut into 0.3-1.0 mm pieces. The tissue pieces were placed in medium containing 1.5M PROH in PBS with 20% serum for 10 min. at room temperature. They were transferred to cyrogenic vials and cooled at 2°C/min to -8°C. The vials were seeded manually with forceps dipped in liquid nitrogen. After 10 min the cooling was continued at a rate of 0.3°C/min to -30°C and then at a rate of 50°C/min to -150°C. When the samples reached this temperature they were transferred to liquid nitrogen.

The vials were removed from the liquid nitrogen and allowed to come to room temperature for 2 min. They were then placed in a water bath at 30°C until they had thawed. The tissue pieces were transferred to a Petri dish containing 1.0M PROH, 0.1M sucrose and 20% serum in PBS for 5 min. They were then transferred to a solution containing 0.5M PROH, 0.1M sucrose and 20% serum in PBS for 5 min and then to a solution containing 0.1M sucrose with 20% serum in PBS for 10 min. The cells were kept in culture medium.

The results obtained from the above experimental procedures are summarized in Table 2 below.

Table 2: Comparison of Results by Different Methods

Method	Cell Viability after Freeze/Thaw
Propanediol-Sucrose	63%
Glyerol-Yolk Buffer	56%
DMSO	50%
Quick DMSO	33%
DMSO-Heparin	23%
Quick-Glycerol	13%

From Table 2 above, it may be seen that the testicular cells that had been frozen using the propanediol-sucrose method had the highest percentage of viable cells upon thawing than cells frozen using the other methods. The propanediol-sucrose freezing method was significantly less damaging to testicular cells than the DMSO method used by Avarbock et al., 1996 for freezing testicular cells prior to transfer. The propanediol-sucrose method was also shown to be good for freezing human ovarian tissue as described by Hovatta et al. (Hovatta et al., Human Reprod. 11: 1268-1272 (1996a), the relevant part of which is incorporated herein by

CLAIMS

1. An in vivo method of incorporating a polynucleotide into a male vertebrate's germ cells, comprising

administering to a male vertebrate's gonads a transfection mixture comprising at least one polynucleotide encoding a desired trait or product, and at least one transfecting agent, and optionally a genetic selection marker, and under conditions effective to reach the vertebrate's germ cells or precursors thereof; and

allowing the polynucleotide encoding a desired trait or product to be taken up by, and released into, the germ cells or precursors thereof.

2. The method of claim 1, further comprising allowing the incorporation of the released polynucleotide into the genome of the germ cells.

3. The method of claim 1 wherein the transfecting agent is selected from the group consisting of liposomes, viral vectors, transferrin-polylysine enhanced viral vectors, retroviral vectors, lentiviral vectors, and uptake enhancing DNA segments, or comprises a mixture of any members of said group.

4. The method of claim 3, wherein the transfecting agent comprises a viral vector selected from the group consisting of retroviral vectors, adenoviral vectors, transferrin-polylysine enhanced adenoviral vectors, human immunodeficiency virus vectors, lentiviral vectors, Moloney murine leukemia virus-derived vectors, mumps vectors, and virus-derived DNAs that facilitate polynucleotide uptake by and release into the cytoplasm of germ cells, or comprises an operative fragment of- or a mixture of any members of said group.

5. The method of claim 1, wherein the transfecting agent comprises an adenovirus vector having endosomal lytic activity, and the polynucleotide is operatively linked to the vector.

6. The method of claim 1, wherein the transfecting agent comprises a lipid transfecting agent.

7. The method of claim 1, wherein the transfecting agent further comprises a male-germ-cell-targeting molecule.

8. The method of claim 7, wherein the male-germ-cell-targeting molecule is specific for targeting spermatogonia, and is a c-kit ligand.

9. The method of claim 1, where the transfection mixture further comprises an immunosuppressing agent.

10. The method of claim 9, wherein the immunosuppressing agent is selected from the group

wherein the polynucleotide encoding a desired trait or product is derived from any genome.

25. The non-human transgenic vertebrate of claim 24, comprising native germ cells carrying in their genome at least one xenogeneic polynucleotide.

26. The non-human transgenic vertebrate of claim 25, wherein the polynucleotide comprises at least one biologically functional gene.

27. The non-human transgenic vertebrate of claim 24, being a male.

28. The progeny resulting from breeding the non-human transgenic vertebrate of claim 27, with a female of the same species.

29. A non-human vertebrate, carrying in its germ cells at least one xenogeneic polynucleotide sequence, said non-human vertebrate being obtained by breeding the vertebrate of claim 24, or progeny thereof, with a member of the opposite sex of the same species, and selecting the bred progeny for the presence of the transfected xenogeneic polynucleotide.

30. The non-human vertebrate of claim 29, which is selected from the group consisting of mammals and birds.

31. The non-human vertebrate of claim 30, which is a mammal selected from the group consisting of humans and non-human primates, canines, felines, swine, farm and marine mammals, pachyderms, equines, murine, ovines and bovine, or a bird selected from the group consisting of ducks, geese, turkeys and chickens.

32. The vertebrate of claim 31, wherein the mammal is selected from the group consisting of wild and domesticated mammals.

33. The vertebrate of claim 31, wherein the mammal is a farm or marine animal.

34. The vertebrate of claim 30, wherein the mammal is selected from the group consisting of a bull and a pig, and the bird is a chicken.

35. A germ cell, obtained from the vertebrate of claim 25.

36. Vertebrate male germ cells, obtained by a method comprising the method of claim 1; raising the transfected male vertebrate; and collecting male germ cells produced by the male vertebrate.

37. The vertebrate male germ cells of claim 36, wherein the method for obtaining them further

of liposomes, viral vectors, transferrin-polylysine enhanced viral vectors, retroviral vectors, lentiviral vectors, and other uptake enhancing DNA segments, or comprises a mixture of any members of said group.

48. The method of claim 47, wherein the transfecting agent comprises a viral vector selected from the group consisting of retroviral vectors, adenoviral vectors, transferrin-polylysine enhanced adenoviral vectors, human immunodeficiency virus vectors, lentiviral vectors, Moloney murine leukemia virus-derived vectors, mumps vectors, and virus-derived DNAs that facilitate polynucleotide uptake by and release into the cytoplasm of germ cells, or said transfecting agent comprises an operative fragment of- or mixture of any members of said group.

49. The method of claim 47, wherein the transfecting agent comprises an adenovirus vector having endosomal lytic activity, and the polynucleotide is operatively linked to the vector.

50. The method of claim 41, wherein the polynucleotide encoding a desired trait or product is in the form of a complex with a viral vector.

51. The method of claim 41, wherein the transfecting agent comprises a lipid transfecting agent.

52. The method of claim 42, wherein the transfecting agent further comprises an agent selected from the group consisting of a male-germ-cell-targeting molecule and at least one genetic selection marker.

53. The method of claim 52, wherein the male-germ-cell-targeting molecule is specifically targeted to spermatogonia and comprises a c-kit ligand; and

the genetic selection marker comprises a gene encoding a detectable product, expression of said gene being driven by a spermatogonia-specific promoter, said promoter being selected from the group consisting of c-kit promoter, b-Myb promoter, c-raf-1 promoter, ATM (axataia-telangiectasia) promoter, RBM (ribosome binding motif) promoter, DAZ (deleted in azoospermia) promoter, XRCC-1 promoter, HSP 90 (heat shock gene) promoter, and FRMI (from fragile X site) promoter.

54. The method of claim 41, wherein the vertebrate is a mammal.

55. The method of claim 54, wherein the mammal is a human.

56. The method of claim 54, wherein the mammal is selected from the group consisting of human and non-human primates and farm and marine mammals.

57. The method of claim 42, wherein the polynucleotide encoding a desired trait or product is derived from the same species of vertebrate as the recipient vertebrate.

70. The non-human vertebrate of claim 67, wherein the mammal is a farm or marine mammal.

71. The non-human vertebrate of claim 68, wherein the mammal is a bull.

72. The non-human vertebrate of claim 68, wherein the mammal is a pig.

73. The non-human vertebrate of claim 66, which is selected from the group consisting of wild and domesticated animals.

74. A germ cell obtained from a vertebrate of claims 24 or 61 comprising a native germ cell carrying in its genome at least one xenogeneic polynucleotide.

75. Vertebrate semen comprising the germ cell of claim 74.

76. A gene therapy method, comprising the method of claim 42, wherein the polynucleotide encoding a desired trait or product is derived from the same species of vertebrate as the recipient vertebrate.

77. A non-human transgenic vertebrate produced by the method of claim 42, wherein the polynucleotide encoding a desired trait or product is derived from any genome.

78. An in vitro method of incorporating at least one polynucleotide encoding a desired trait into a maturing male germ cell, comprising

obtaining a maturing male germ cell from a vertebrate;

transfecting the germ cell in vitro with at least one polynucleotide encoding a desired trait in the presence of a gene delivery mixture comprising at least one transfecting agent, and optionally a polynucleotide encoding a genetic selection marker, at about or below the vertebrate's body temperature and for a transfection-effective period of time; and

allowing the polynucleotide encoding a desired trait to be taken up by, and released into the germ cell.

79. The method of claim 78, further comprising allowing the incorporation of the released polynucleotide into the genome of the germ cell.

80. The method of claim 78, wherein the encoding a desired trait is incorporated into the vertebrate germ cell's genome.

81. The method of claim 78, wherein the maturing male germ cell comprises a spermatogonia or other undifferentiated male germ cell.

82. The method of claim 78, wherein the transfection is conducted under conditions of temperature

90. The method of claim 78, wherein the vertebrate is a mammal.

91. The method of claim 90, wherein the mammal is a human.

92. The method of claim 90, wherein the mammal is selected from the group consisting of human and non-human primates and farm and marine mammals.

93. The method of claim 78, wherein the polynucleotide encoding a desired trait is derived from the same vertebrate species as the maturing germ cell.

94. The method of claim 78, wherein the vertebrate is selected from the group consisting of wild and domesticated vertebrates.

95. The method of claim 78, wherein the polynucleotide encoding a desired trait is derived from a mammal selected from the group consisting of human and non-human primates, canines, felines, swines, farm mammals, pachyderms, marine mammals, equines, murine, ovine and bovine, or from a bird selected from the group consisting of ducks, geese, turkeys and chickens.

96. The method of claim 95, wherein the polynucleotide is derived from a human.

97. A non-human transgenic vertebrate, or its progeny, comprising a native germ cell carrying in its genome at least one xenogeneic polynucleotide, said polynucleotide having been incorporated into the genome of said germ cell through the method of claim 78.

98. The non-human transgenic vertebrate of claim 97, wherein the polynucleotide comprises at least one biologically functional gene.

99. The non-human transgenic vertebrate of claim 98, being a male.

100. The non-human transgenic vertebrate of claim 99, harboring native male germ cells transfected with a xenogeneic polynucleotide.

101. The progeny resulting from breeding the non-human transgenic vertebrate of claim 99 or progeny thereof, with a female of the same species.

102. A non-human vertebrate, carrying in its germ cells at least one xenogeneic polynucleotide sequence, said vertebrate obtained by breeding the vertebrate of claim 98 or progeny thereof, with a member of the opposite sex of the same species, and selecting the bred progeny for the presence of the transfected xenogeneic polynucleotide.

116. The kit of Claim 114, wherein the transfecting agent comprises a viral vector selected from the group consisting of retroviral vectors, adenoviral vectors, transferrin-polylysine enhanced adenoviral vectors, human immunodeficiency virus vectors, lentiviral vectors, Moloney murine leukemia virus-derived vectors, mumps vectors, DNAs that facilitate polynucleotide uptake by and release into the cytoplasm of germ cells, or comprises an operative fragment of- or mixture of any members of said group.

117. The kit of Claim 114,wherein the transfecting agent comprises an adenovirus vector having endosomal lytic activity, and the polynucleotide is operatively linked to the vector.

118. The kit of Claim 114, wherein the transfecting agent comprises a lipid transfecting agent.

119. The kit of Claim 114, wherein the transfecting agent further comprises a male-germ-cell-targeting molecule.

120. The kit of Claim 119, wherein the male-germ-cell-targeting molecule is specific for targeting spermatogonia and comprises a c-kit ligand.

121. The kit of Claim 114, where the transfection mixture further comprises an immunosuppressing agent.

122. The kit of Claim 121, wherein the immunosuppressing agent is selected from the group consisting of cyclosporin and corticosteroids.

123. The kit of Claim 119, wherein the male-germ-cell-targeting molecule is specifically targeted to spermatogonia and comprises a c-kit ligand; and

the genetic selection marker comprises a gene expressing a detectable product driven by a spermatogonia-specific promoter.

124. The kit of Claim 119, wherein the male-germ-cell-targeting molecule is specifically targeted to spermatogonia and comprises a c-kit ligand; and

the genetic selection marker comprises a gene expressing a detectable product, driven by a spermatogonia-specific promoter, said promoter selected from the group consisting of c-kit promoter, b-Myb promoter, c-raf-1 promoter, ATM (axataia-telangiectasia) promoter, RBM (ribosome binding motif) promoter, DAZ (deleted in azoospermia) promoter, XRCC-1 promoter, HSP 90 (heat shock gene) promoter, and FRMI (from fragile X site) promoter.

125. The kit of Claim 114, wherein at least one polynucleotide comprises at least one polynucleotide sequence encoding a genetic selection marker.

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/24238

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/89 C12N15/86 C12N15/88 C12N5/06 A01K67/027 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP 0 867 114 A (HOECHST MARION ROUSSEL LTD (JP)) 30 September 1998 see the whole document	1-4,6, 11, 24-39, 114-116, 118
X	-& WO 97 11597 A3 April 1997	1-4,6, 11, 24-39, 114-116, 118

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
20 April 1999	06/05/1999

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Int ional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92 03459 A (SLOAN KETTERING INST CANCER) 5 March 1992 see page 1, line 1 - page 7, line 12 see page 27, line 21 - line 28	7,8,52, 53,88, 89,119, 120

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 7 24-30 52 61-73 88 97-109 119, all partially

Claims 7, 52, 88 and 119 have been searched only incompletely, because they relate to agents, defined by the result to be achieved. These claims therefore are unclear and lack technical disclosure and thus do not fulfill Article 6 PCT (see also PCT Search Guidelines, Chapter III, 3.7)

Claims 24-30, 61-73 and 97-109 have been searched only incompletely because they are unduly speculative, beyond the extend to which they are supported by the description. These claims therefore lack technical disclosure and thus do not fulfill Article 6 PCT (see also PCT Search Guidelines, Chapter III, 3.7)